Chapter 13

Summary, discussion and future perspectives

Summary and discussion

Glioblastoma (GBM), a highly malignant brain neoplasm, represents one of the most complex types of cancer. The diagnosis GBM leaves patients with a dismal prognosis and limited therapeutic options are available, which all can only transiently halt disease progression. Better understanding of the genetic machinery that results in this devastating disease is hoped to improve these perspectives and over the last decade the molecular classification of GBMs has moved on apace. During the initiation of the studies described in the thesis, molecular subclasses based on transcriptional profiles had just been identified [1-3], and a vast majority of studies evaluating the druggable targets within these subclasses was underway.

With **Part I** of the thesis we have aimed to contribute to this understanding by performing several studies on GBM tumor material that was surgically resected from patients. First, we explored the possibility of molecular subclassification of GBMs in **Chapter 3** on tumor material that is already routinely obtained for diagnosing patients, and with the use of only widely used pathological methods. To prevent using up the available tissue and to allow for the analyses of an extensive panel of markers, we created a tissue microarray that contained GBM tumor tissue from 167 patients. Through the application of primarily immunohistochemical stainings, we were able to subclassify two-thirds of the tumors into proneural (PN), classical (CLAS) and mesenchymal (MES) subclasses.

In **Chapter 5** and **Chapter 6** we assessed the influence of environmental stimuli on subclass identity of GBM tumor cells. Glioma stem-like cells (GSCs) are endowed with tumor-initiating properties [4] and some studies have shown the role of GSCs in clinical relapse due to therapy refractoriness [5-7]. Microenvironmental support is provided to the GSCs as they reside in their preferential perivascular niche [8], and we showed that both TGF-β and hypoxia can induce the expression of MES markers in glioma cell models. This should be interpreted as malignant progression, since a PN-to-MES transition has been observed at disease recurrence and the MES phenotype has been reported to mediate radio-resistance [2,7]. These studies indicate that environmental cues, as we also discussed extensively in **Chapter 2**, can contribute significantly to GBM progression.

Due to extensive associations between receptors with tyrosine kinase domains and the above-described molecular subclasses, we explored kinase activity profiles of molecular subclasses in **Chapter 4**. Unexpectedly, only few phosphorylation sites on the array were found to associate with molecular subclasses, irrespective of the mutational status of, for example, GBMs of the CLAS subtype. This suggests that although receptor expression levels are aberrant in subgroups of tumors, substantial ongoing cross-talk between multiple signaling routes in GBMs prevented the identification of canonically exploited signaling pathways. It is also possible that the substantial percentage of non-tumor cells in GBM tissue lysates desensitized the array, and together these findings complicate therapeutic target selection in GBM further.

Part II of the thesis then shifted focus to the role of angiogenesis in GBM. The examination

of vascular parameters in **Chapter 8** on GBM tissue sections indicated that the ascribed angiogenic profile to the MES subclass could have been confounded by the fact that these tumors had a similar number of vessels, but that these were merely larger in size. It should be noted that the functional interpretation would benefit greatly from insight into the perfusion status, because pro-thrombotic mechanisms [9], vascular normalization [10,11] and edema [10] represent significant modifiers of vascular effects.

In **Chapter 9** we subsequently identified IL-8 as a potentially important mediator of the vascular enlargement that we observed in MES GBM tissue from patients. IL-8 was substantially higher expressed in pro-angiogenic tumor, MES GSC lines and the addition of an IL-8 neutralizing antibody only reduced angiogenesis when it was added to the conditioned medium of MES GSCs. It could however be noted that the *in vitro* results in this chapter are limited in effect size, but the differences in, for example, *in vitro* serum requirements between cell lines severely impeded absolute quantitation of paracrine effects and could have prevented the identification of actual larger effect sizes. The exploration of IL-8 inhibition in an *in vivo* xenograft model comparing the effect of treatment on engrafted PN and MES GSCs could substantiate our findings further.

In contrast to the microenvironmental approach adopted in the studies where subclass plasticity was assessed, we next examined the effects of key angiogenic mediators with a strong focus on tumor cell altering effects instead. Angiopoietin-2 (Ang-2) and VEGFA, well-known for their pro-angiogenic effects in glioma angiogenesis [12], exerted an unexpected indirect anti-angiogenic effect when they were used to stimulate tumor cells *in vitro* as is described in **Chapter 10**. *In vivo* stimulation with these agents, however, did not alter tumor growth or vascularization parameters, potentially signifying the inability of angiogenesis modulation in xenograft GBM models. In contrast, the inhibition of Ang-2 and VEGFA in **Chapter 11** in the exact same model did result in reduced expression of vascular parameters.

The discrepancies between these studies could potentially be explained by several factors. First, the fact that an anti-angiogenic effect with conditioned medium from GBM cells is observed relative to the control PBS-stimulation condition, perhaps needs to be rephrased to reduced pro-angiogenic effects of GBM cells on endothelium. Second, the expression analyses of angiogenic signaling molecules in **Chapter 8** and **Chapter 9** illustrate that a multitude of signaling routes are exploited in GBMs, which allows the xenografts to already reach a vascular saturation stage even before application of our treatments. It is possible that the selection of this xenograft GBM model prevented the detection of the anti-angiogenic properties of Klotho in **Chapter 12**.

Two recent reports allow us to compare the combined Ang-2 and VEGFA inhibition that we performed in our immunocompromised model to studies that were performed in immunocompetent GBM models. The reports of the other studies indicated that the recruitment of macrophages and the phenotypical transition of these following combined Ang-2 and VEGFA inhibition served a crucial role in the survival extension that was achieved in their *in vivo* studies [13,14]. Since we were unable to identify an effect on tumor growth or survival, we conclude that the immunocompromised environment under which our *in vivo* experiment was performed likely contributed substantially to treatment outcome. The multifaceted properties of both Ang-2 and VEGFA are also extensively reviewed in **Chapter 7**, and we speculate that

the pro-inflammatory effects of especially Ang-2 likely have more potential in an anti-tumor approach than the combined anti-angiogenesis approach that was employed by us [13-15].

Future perspectives

Although we want to acknowledge the substantial advancements in our understanding of the elusive complexity and molecular heterogeneity of GBMs by The Cancer Genome Atlas initiative and associated groups, the therapeutic implications of the extensive molecular classification have thus far not been groundbreaking. Apart from selective benefit from the anti-angiogenic drug Bevacizumab in PN tumors that do not harbor a mutation in the *isocitrate dehydrogenase 1* gene (*IDH1*, [16]), to our knowledge no selective therapeutic benefit of molecular GBM subclasses has been reported. An ongoing clinical trial that specifically focuses on *epidermal growth factor receptor* (*EGFR*) amplification, an aberration associated with the transcriptionally described CLAS subgroup, is another example of a trial that is at an advanced clinical stage, but notably also addresses a specific molecular aberration and not an expression pattern. Clinically a tendency can thus be observed that specific aberrations are targeted instead of an expression signature. Additionally, the field has progressed with the identification of molecular heterogeneity of GBMs in methylation patterns [17] and genomic profiles that independently resulted in an alternative GBM classification [18]. The aberrations described in the latter study have recently been adopted by the new WHO guidelines [19] and studies to be initiated will very likely focus on these aberrations.

By focusing on specific molecular aberrations as described in the 2016 WHO guidelines, it is ensured that tumor specific alterations are studied, given that molecular aberrations in these tumors generally do not concern somatic mutations. The increase in GBM incidence with age also argues for that notion [20]. The morphometrical analyses of the tumor vasculature in this thesis and the high percentage of myeloid cell content in freshly isolated biopsies both call into question whether transcriptional profiling of whole GBM tissue that exhibits such cellular heterogeneity is appropriate and in many ways has allowed us to appreciate another dimension of multiform in GBM. Lower tumor cell percentages could have masked many small but important tumor specific properties.

The discordant results obtained with *in vivo* experimentation in this thesis shed light on the important differences between *in vitro*, *in ovo* and *in vivo* models that were employed for our experiments. It underscores the artificial aspect of *in vitro* culturing, as we are, for example, simply unable to define whether conditioned medium from GBM tumor cells exerted a pro-angiogenic effect due to the extensive set of experimental parameters that are controlled. Unfortunately this controlled experimental environment is not inspired by physiological or clinical considerations, but merely to allow for the observation of consistent effects. Improved *in vitro* models that would allow more physiological (co-)culturing conditions could help us gain insight into the crucial interactions that ultimately contribute to tumor progression. Complementary approaches involving both clinically relevant xenograft models alongside

immunocompetent transgenic mouse models will be needed to reach definitive conclusions on the role the microenvironment in GBM.

As a concluding remark, I believe that the field could advance by investing more in the study of therapy refractoriness. The distressing situation that patients with GBM undergo fully justifies the exploration of novel, currently unapproved therapies for this disease. From a research perspective, however, a balance is required between the exploration of novel promising agents and the attempts to understand why other agents did not provide benefit, because the latter could very well be our next step to improved therapeutic efficacy. To accomplish this, advances in understanding of basic tumor biology, plasticity, therapy response and resistance are fundamental.

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